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Gp96 is a receptor for a novel *Listeria monocytogenes* virulence factor, Vip, a surface protein

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By comparative genomics, we have identified a gene of the intracellular pathogen *Listeria monocytogenes* that encodes an LPXTG surface protein absent from nonpathogenic *Listeria* species. This gene, *vip*, is positively regulated by PrfA, the transcriptional activator of the major *Listeria* virulence factors. Vip is anchored to the *Listeria* cell wall by sortase A and is required for entry into some mammalian cells. Using a ligand overlay approach, we identified a cellular receptor for Vip, the endoplasmic reticulum (ER) resident chaperone Gp96 recently shown to interact with TLRs. The Vip–Gp96 interaction is critical for bacterial entry into some cells. Comparative infection studies using oral and intravenous inoculation of non-transgenic and transgenic mice expressing human E-cadherin demonstrated a role for Vip in *Listeria* virulence, not only at the intestine level but also in late stages of the infectious process. Vip thus appears as a new virulence factor exploiting Gp96 as a receptor for cell invasion and/or signalling events that may interfere with the host immune response in the course of the infection.

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Introduction

Listeria monocytogenes is an intracellular food-borne pathogen that causes listeriosis, an infection characterised by gastroenteritis, meningitis, encephalitis and maternofetal infections in humans. *L. monocytogenes* enters the host via contaminated foods, invades the small intestine, translocates to mesenteric lymph nodes and spreads to the liver, spleen, brain and, in pregnant women, the placenta. This bacterium has the ability to cross three tight barriers: the intestinal, the blood–brain and the placental barriers. During infection, it enters, survives and multiplies inside phagocytic and non-phagocytic cells (Khelef *et al*, 2005).

Intracellular pathogens have developed a variety of strategies to induce their entry into nonphagocytic mammalian cells, implicating both bacterial and host cell components. In the case of *Salmonella* or *Shigella*, entry is mediated by type III secretion systems allowing direct activation of cytoskeleton components by intracellular delivery of bacterial effectors. Entry of *Yersinia* or *Listeria* implies a direct interaction between bacterial ligands and mammalian receptors. For *Yersinia*, the outer membrane protein invasin binds to β_1 integrin receptors (Cossart and Sansonetti, 2004). Entry of *L. monocytogenes* is mediated by several ligand–receptor interactions that mediate adhesion and invasion. The known adhesins include FbpA that interacts with fibronectin (Dramsi *et al*, 2004), the autolysin Ami (Milohanic *et al*, 2001), ActA that may promote attachment via proteoglycans (Suarez *et al*, 2001), the 104 kDa Lap that binds the heat shock protein Hsp60 (Wampler *et al*, 2004) and, most importantly, internalin (InlA), which is a ligand for E-cadherin (Mengaud *et al*, 1996b). The two major *L. monocytogenes* invasins are InlA and InlB. InlB interacts with three receptors—c-Met, the hepatocyte growth factor receptor (Shen *et al*, 2000); gC1q-R, a ubiquitous glycoprotein (Braun *et al*, 2000); and proteoglycans (Jonquieres *et al*, 2001).

Whereas the infectious process has been studied extensively in cultured cells (Cossart and Lecuit, 1998), the *in vivo* infection and the bacterial factors involved in the successive steps that lead to listeriosis are less precisely known. Several animal models have been used to dissect the *Listeria* infection. Following oral inoculation, *L. monocytogenes* induces in guinea-pig a gastroenteritis resembling that observed in humans and, after crossing of the intestinal barrier, induces a dose-dependent lethality following systemic dissemination (Lecuit *et al*, 2001). Due to a species specificity of the InlA/E-cadherin interaction—InlA does not interact with mouse E-cadherin—and to investigate the role of InlA *in vivo*, a transgenic mouse model (*hEcad*) was generated. Enterocytes of *hEcad* mice allowed efficient entry of *L. monocytogenes*, subsequent crossing of the intestinal barrier, bacterial multiplication in the small intestine and dissemination to target organs. Guinea-pigs and transgenic mice are the most permissive animal models to orally acquired listeriosis. In normal mice, intravenous inoculation of *L. monocytogenes* induces a dose-dependent lethality. This infection model has been widely and successfully used for the analysis of the role of virulence factors implicated in systemic listeriosis.

Following the determination of the *Listeria* genome sequence (Glaser *et al*, 2001), we undertook to identify new virulence factors. Among possible bacterial factors interacting with host tissues and involved in virulence, surface

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proteins are privileged candidates. Many of these surface proteins (LPXTG proteins) are anchored to the cell wall via a C-terminal sorting signal reaction catalysed by sortase A (SrtA) (Dhar *et al*, 2000; Bierne *et al*, 2002). As said above, InlA, the first *Listeria* LPXTG protein identified, plays *in vivo* a key role in crossing the intestinal and the placental barriers (Lecuit *et al*, 2001, 2004). In addition to InlA, the *L. monocytogenes* EGDe genome encodes 40 other putative LPXTG proteins (Cabanes *et al*, 2002), among which are InlE, InlF, InlG and InlH whose exact function *in vivo* remains unknown (Dramsi *et al*, 1997; Raffelsbauer *et al*, 1998; Schubert *et al*, 2001). Of the 41 *L. monocytogenes* LPXTG proteins, 20 have no orthologue in the nonpathogenic and noninvasive strain *L. innocua* and are thus candidates to be implicated in *Listeria*–host interactions and virulence.

We focused on *lmo0320*, an *L. monocytogenes* LPXTG surface protein encoding gene absent from all nonpathogenic *Listeria* species, and provide here evidence for the implication of Lmo320, renamed Vip for virulence protein, in *Listeria* virulence. We identify the endoplasmic reticulum (ER) resident chaperone Gp96 as a Vip cellular receptor and demonstrate that the Vip–Gp96 interaction is critical for *Listeria* entry into some mammalian cells and for infection *in vivo*.

Results

Identification of a new L. monocytogenes LPXTG surface protein

Lmo0320 is one of the 20 *L. monocytogenes* putative LPXTG proteins identified as absent from the nonpathogenic species *L. innocua* (Cabanes *et al*, 2002). *lmo0320* is predicted to encode a protein of 399 amino acids (predicted molecular mass ≈43 kDa) containing a signal sequence and a C-terminal sorting signal (Figure 1A). Among the 41 *L. monocytogenes* LPXTG proteins, 19 contain, as InlA, a leucine-rich repeat domain. Lmo0320 does not belong to the Internalin family but contains as a most remarkable feature a proline-rich region (amino acids 268–318). Lmo0320 has been named Vip, for virulence protein. In the genome of *L. monocytogenes*, *vip* is flanked by *lmo0319* and *lmo0321*, predicted to encode a phospho-beta-glucosidase and a protein of unknown function, respectively (Figure 1B). In the *L. innocua* genome, *vip* is replaced by a small ORF (*lin0345*) encoding a putative 63-amino-acid polypeptide with no signal peptide and no similarity with Vip or other proteins or domains in databases. *lin0344* and *lin0346* are orthologues of *lmo0319* and *lmo0321*. DNA hybridisation carried out on 113 *Listeria* strains revealed that *vip* is an *L. monocytogenes*-specific gene: *vip* is always present in *L. monocytogenes* strains of lineages I (serovars 1/2a, 1/2c, 3a, 3c) and II (serovars 1/2b, 3b, 4b, 4d, 4e, 7), absent from the rare lineage III (serovars 4a, 4c) and absent from all the *L. innocua* strains tested as well as from all the other *Listeria* species (*L. ivanovii, L. seeligeri, L. welshigeri* and *L. grayi*) (Doumith *et al*, 2004).

vip is positively regulated by PrfA

The transcriptional activator PrfA positively regulates most of the *L. monocytogenes* virulence genes so far identified (Dussurget *et al*, 2002; Milohanic *et al*, 2003). The role of PrfA in the transcription of *vip* was assessed by RT–PCR and Northern blot. RNAs were isolated from *L. monocytogenes* and its isogenic *prfA* mutant during exponential growth in BHI at 37°C. We used *hly* as a control gene positively regulated by PrfA and *iap* as a control gene not PrfA regulated. As shown by RT–PCR (Figure 2A), the levels of *vip* mRNA were lower in the Δ*prfA* strain, demonstrating that PrfA regulates *vip* transcription. Compared to *hly* and *iap*, *vip* was expressed at lower levels in BHI at 37°C. No RT–PCR product was detected in controls lacking reverse transcriptase, demonstrating the absence of DNA contaminating the RNA preparations (data not shown). Northern blot confirmed that *vip* is positively regulated by PrfA (Figure 2B). Preliminary results indicate that *vip* expression, as all major virulence factors, is thermoregulated (data not shown).

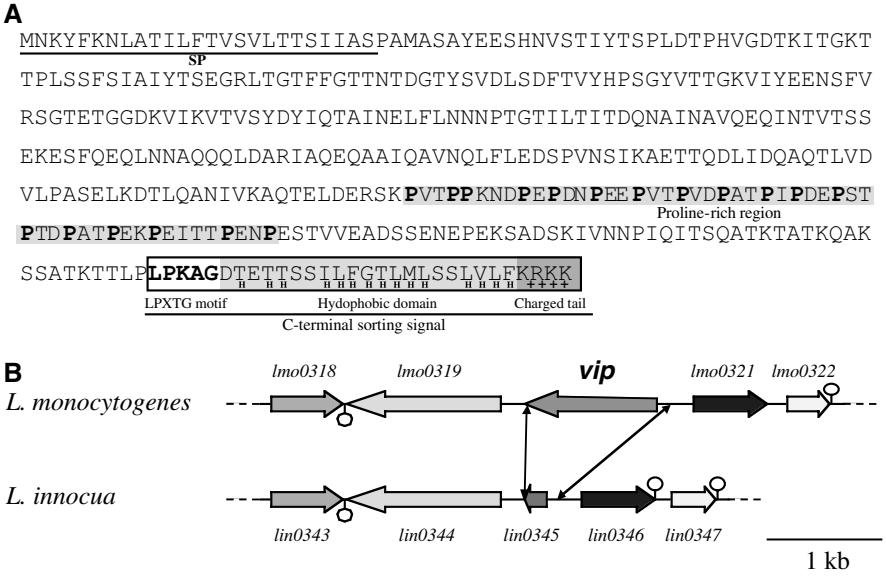

Figure 1 (**A**) Amino-acid sequence of the *vip* product. Sp = signal peptide. (**B**) Genomic organisation of the *vip* region in *L. monocytogenes* and comparison with the homologous region in *L. innocua*. The arrows indicate gene orientation and hairpins putative terminators.

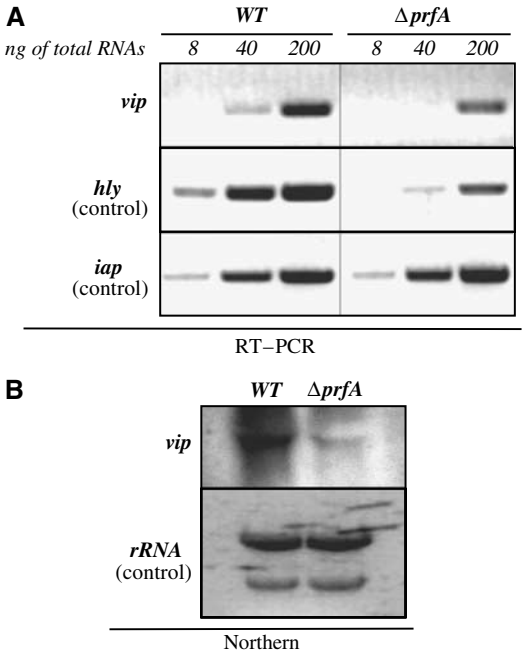

Figure 2 *vip* is PrfA regulated. (**A**) RT–PCRs of RNAs from logarithmic cultures in BHI at 37°C of *L. monocytogenes* WT and Δ*prfA* strains. The *hly* gene was used as control PrfA-regulated gene and the PrfA-independent *iap* gene to control RNA amounts. (**B**) Northern blot analysis of RNAs isolated from logarithmic cultures in BHI at 37°C of *L. monocytogenes* WT and Δ*prfA* strains. rRNAs were used to control RNA amounts.

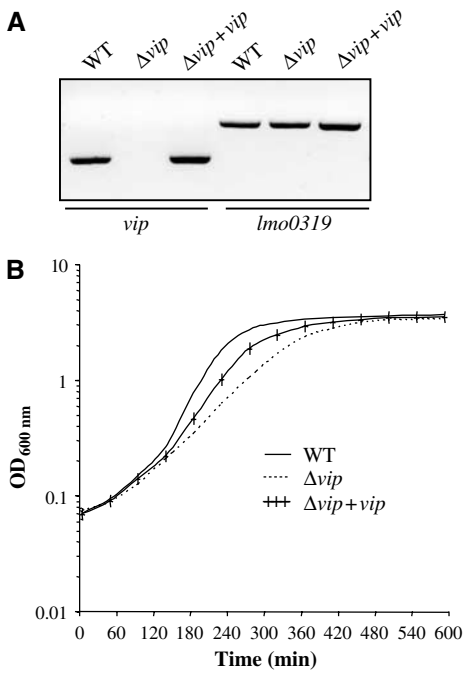

Figure 3 Gene replacement of *vip*. (**A**) RT–PCR on total RNAs from *L. monocytogenes* WT and Δ*vip* strains using specific primers of *vip* and *lmo0319*. Fragments (*vip* and *0319*) amplified are visualised. RT–PCR was performed on *L. monocytogenes* Δ*vip* + *vip* strains to confirm *vip* expression in the complemented strain. (**B**) Growth curves in BHI at 37°C.

Vip is a surface protein anchored to the bacterial cell wall by sortase A

In order to analyse the surface localisation of Vip and study its potential role in virulence, we constructed an isogenic *vip*-deletion mutant (Δ*vip*). Replacement of *vip* was confirmed by Southern blot, PCR (data not shown) and RT–PCR (Figure 3A). RT–PCR performed using specific primers for *vip* and the downstream *lm0319* gene revealed that the gene replacement had no polar effect on *lmo0319* expression. Insertion of the *vip* wild-type (WT) gene as a single copy under its own promoter on the chromosome of the *vip* mutant, at the PSA bacteriophage attachment site using the integration vector pPL2 (Lauer *et al*, 2002), restored *vip* expression (Figure 3A). As compared to the WT strain, no phenotypic differences were detected for Δ*vip* with respect to haemolytic activity, expression of the main *Listeria* virulence factors (InlA, InlB, ActA, LLO) (data not shown) and microscopic morphology (Figure 4). The growth rate of Δ*vip* in BHI at 37°C was lower than that of the WT, reaching the same bacterial density at the stationary phase (Figure 3B). This phenotype was restored, albeit partially, in the complemented strain.

We predicted from its amino-acid sequence (Figure 1A) that Vip would be expressed on the bacterial surface. To test this hypothesis, a Vip recombinant protein was produced and a Vip-specific polyclonal antibody was generated. Immunofluorescence microscopy with anti-Vip antibody demonstrated the localisation of Vip on the bacterial surface (Figure 4). The anti-Vip immunoreactive protein was absent from the surface of Δ*vip*, but detected at the surface of the Δ*vip* complemented strain, demonstrating the specificity of the anti-Vip antibody. The presence of InlA on the bacterial surface was not affected in Δ*vip* when compared to WT, using InlA-specific antibody, suggesting that anchoring of other surface proteins to the bacterial cell wall is not impaired in the absence of Vip (Figure 4). The transpeptidase SrtA covalently links LPXTG proteins to the Gram-positive bacteria peptidoglycan (Bierne *et al*, 2002). To test if Vip is anchored to the listerial surface by SrtA, we analysed the presence of Vip and InlA on the surface of the Δ*strA* mutant. Similar to InlA, Vip was not detected on the surface of Δ*strA*. These two proteins were still detected on the surface of a mutant for SrtB, the second *Listeria* sortase involved in the attachment of a subset of proteins displaying an NXZTN sorting motif (Bierne *et al*, 2004) (Figure 4). Altogether, these results indicate that Vip is an *L. monocytogenes* surface protein anchored to the bacterial cell wall by SrtA.

Vip is required for entry into some eukaryotic cells

As surface proteins are in contact with the host cell during infection, we first tested whether Vip could be implicated in *Listeria* entry. The WT, Δ*vip* and complemented strains, together with the previously described Δ*inlA* strain (Lingnau *et al*, 1995), were tested for entry into different cells. These cell lines include cells in which the *L. monocytogenes* entry depends on the InlA pathway (human enterocyte-like Caco-2 and guinea-pig epithelial GPC16 cells) or cells in which entry is mostly InlB-dependent (mouse fibroblast L2071 and African green monkey kidney Vero cells). In Caco-2 and L2071 cells, the *vip* mutant was ≈10-fold less invasive than WT (Figure 5A), indicating that Vip plays a significant role in *Listeria* entry into these cells. In contrast, deletion of *vip* had no effect on entry into GPC16 and Vero cells. The entry levels of the complemented strain were comparable

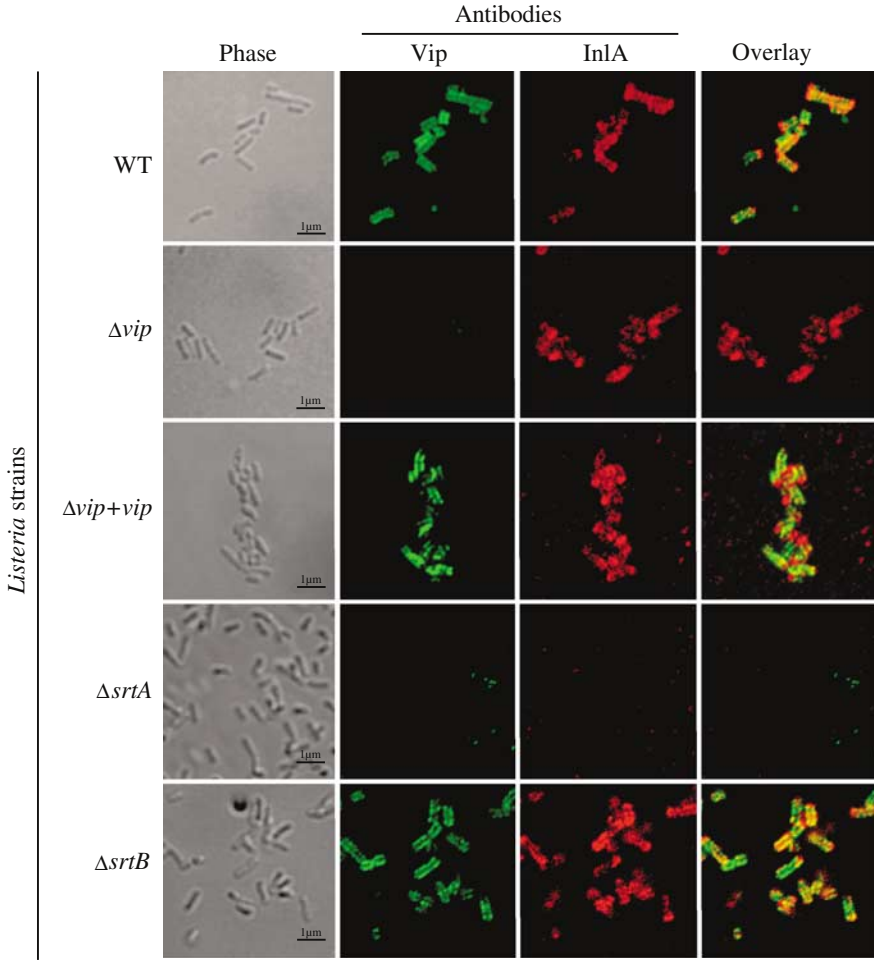

Figure 4 Vip is a surface protein anchored to the bacterial cell wall by SrtA. Morphology of the *vip* mutant and display of Vip and InlA on the bacterial surface of *L. monocytogenes* WT, Δ*vip*, Δ*vip*+*vip*, Δ*srtA* and Δ*srtB* strains were analysed by immunofluorescence staining and confocal microscopy using antibodies against Vip and InlA.

to those of WT, confirming that the entry defect phenotype of the *vip*-deletion mutant resulted from direct mutation of the locus (Figure 5A). Taken together, these results suggest that *vip* is required for entry into Caco-2 and L2071 cells.

Intracellular multiplication of the WT, Δ*vip* and complemented strains after internalisation was studied in L2071 cells. As shown in Figure 5B, the three strains grew with similar multiplication rates after internalisation, indicating that the slight growth delay observed for Δ*vip* in BHI at 37°C has no consequences on intracellular multiplication. Entry and behaviour of Δ*vip* were also analysed in the murine macrophage cell line J774. As compared to the WT strain, entry and intracellular survival of Δ*vip* were not impaired in J774 cells (data not shown).

We also examined whether the *vip* mutant was affected in adherence properties, intracellular motility and cell-to-cell spread in L2071 cells. The WT and Δ*vip* bacteria adhered and formed similar actin tails and plaques of the same size on cultured cell monolayers (data not shown), indicating that deletion of *vip* did not alter adherence, intracellular motility and cell-to-cell spread, and strictly results in loss of invasiveness.

Identification of Gp96 as a Vip surface cellular receptor

As bacterial surface factors are likely to interact with host cell surface proteins, we used a ligand overlay approach to detect a putative Vip cellular receptor. Solubilised Caco-2, L2071 and Vero cell proteins separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto membrane were incubated with purified Vip. Bound Vip was detected with anti-Vip antibody. Several protein bands that could act as Vip receptors were detected (Figure 6A). When Vip was omitted from the reaction, all these bands, except one, were still detected, indicating that anti-Vip or secondary antibodies reacted nonspecifically with some proteins. The remaining protein band of ≈100 kDa, only detected in Caco-2 and L2071 reactions, two cell lines in which Vip seems to play a role in *Listeria* entry, could thus correspond to a Vip receptor. The ≈100 kDa band was identified by mass spectrometry. The results revealed exact matches with the human endoplasmic reticulum (ER) chaperone Gp96 in Caco-2 cells and mouse Gp96 in L2071 cells (Figure 6B). To confirm the Vip–Gp96 interaction, pull-down assays were performed on Caco-2 and L2071 total cell lysates using purified Vip. Western blot using anti-Gp96 revealed an ≈100 kDa band (expected size of a Gp96 monomer) (Figure 6A), confirming that Gp96 was a Vip ligand. Control reaction without Vip captured nonspecific proteins but no ≈100 kDa protein.

To test if Vip interacts with surface-exposed Gp96, Caco-2 and L2071 cell surface proteins were labelled using the membrane-impermeable biotinylation reagent sulpho-*N*-

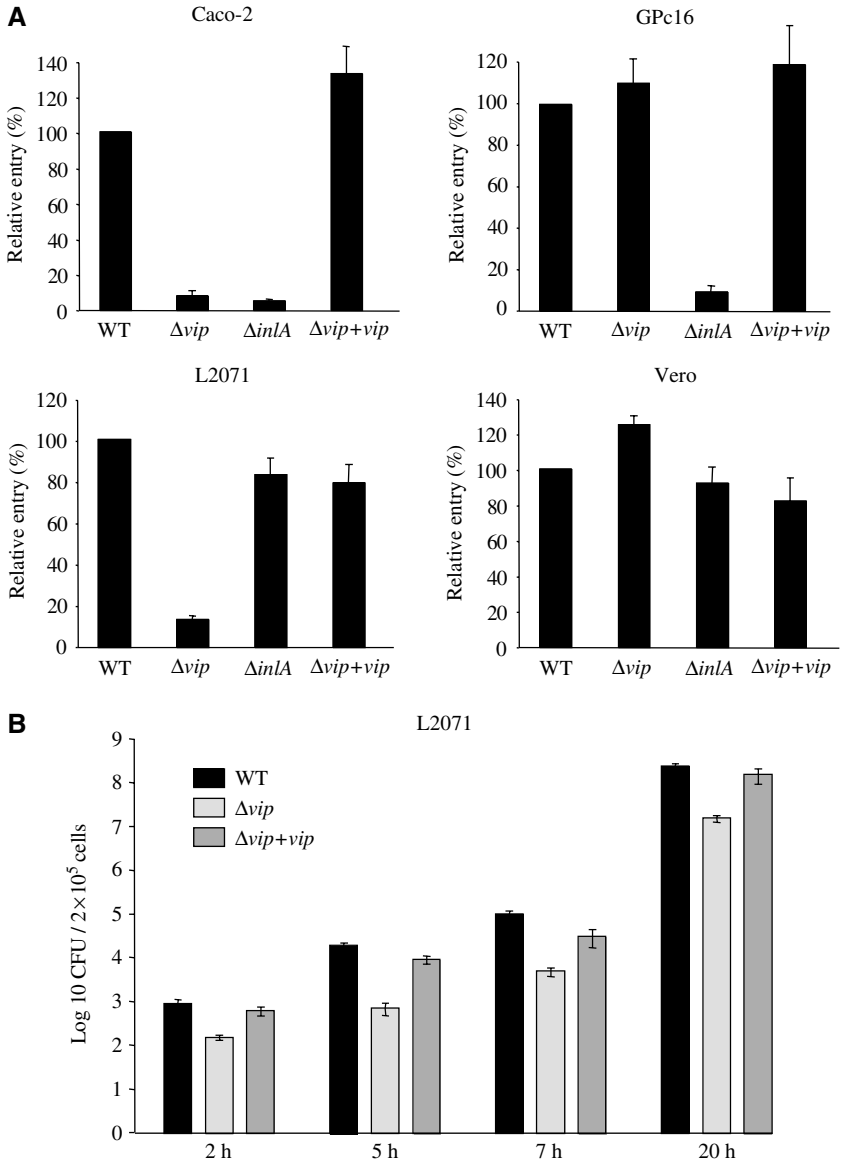

Figure 5 Role of Vip in *Listeria* entry. (**A**) Entry of *L. monocytogenes* WT, Δ*vip*, Δ*inlA* and Δ*vip* + *vip* strains into Caco-2, GPC16, L2071 and Vero cells. After a 1 h infection, invasion values were calculated from the number of bacteria that survived to 2 h gentamicin incubation. Values are given relative to the invasion of the WT strain arbitrarily fixed to 100. (**B**) Entry and intracellular behaviour of the WT, Δ*vip* and Δ*vip* + *vip* strains in L2071 cells. Experiments were repeated three times in duplicate for each cell line.

hydroxysuccinimide (NHS) biotin. Biotinylated proteins were used for pull-down assays with purified Vip, and Vip-precipitated surface proteins were revealed using streptavidin or anti-Gp96. In both Caco-2 and L2071 cells, a band was detected in the biotinylated cell surface protein fraction corresponding to Gp96, as confirmed using anti-Gp96 (Figure 6C). In a control reaction without Vip, Gp96 protein was barely detectable. The possibility that the surface biotinylation may have artifactually labelled intracellular proteins is unlikely, because we could not detect any biotinylated α-catenin (Figure 6C). Altogether, these results identified Gp96 as a cellular receptor for Vip.

Gp96 is expressed at the cell surface

Expression of Gp96 in different cell lines was first analysed by immunoblotting using anti-Gp96 and total cell extracts. Gp96 was expressed in all examined cell lines (Figure 7A). To further assess the cellular localisation of Gp96, we analysed Gp96 distribution in Caco-2, L2071, GPC16 and Vero cells by confocal microscopy. Nonpermeabilised cells were stained with anti-Gp96 followed by secondary antibody and then with phalloidin to label F-actin. In Caco-2 and L2071 cells, Gp96-associated fluorescence was clearly detected at the cell surface of non-permeabilised cells (Figure 7B). In GPC16 and Vero cells, Gp96 was poorly detected at the cell surface. Thus, in addition to cell surface protein biotinylation data, confocal microscopy analysis demonstrated that Gp96 is present on the surface of Caco-2 and L2071, suggesting that in these cell lines surface-exposed Gp96 can interact with Vip, thus promoting *Listeria* entry.

Antibodies raised against Vip or Gp96 block Listeria invasion

To investigate the relevance of Gp96 and of the Gp96–Vip interaction in bacterial internalisation, L2071 cells were

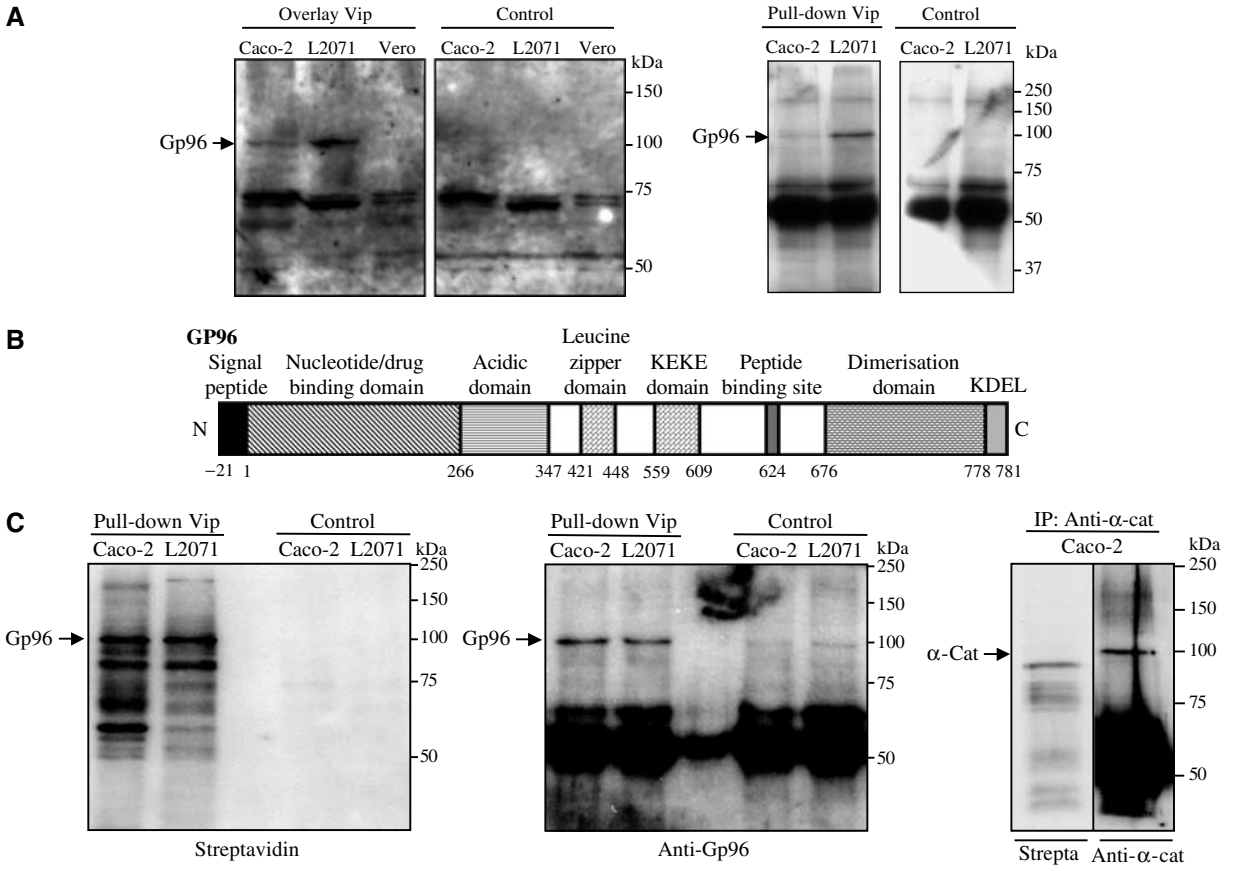

Figure 6 Gp96 is a Vip surface cellular receptor. (**A**) Identification of Vip receptor by ligand overlay assay and confirmation by pull-down assay. For overlay, total cell extracts from Caco-2, L2071 and Vero cells were separated on a 10% polyacrylamide gel, transferred onto a PVDF membrane and probed successively with Vip and anti-Vip and detected with peroxidase-conjugated secondary antibody (Overlay Vip), or only with anti-Vip and secondary antibodies (Control). For pull-down, total cell extracts from Caco-2 and L2071 cells were incubated with Vip and immunoprecipitated with anti-Vip and Sepharose beads (Pull-down Vip). In control assay, the incubation step with Vip was omitted (Control). Captured proteins were separated on a 10% polyacrylamide gel, transferred onto a PVDF membrane and probed with anti-Gp96. (**B**) Schematic representation of Gp96. Gp96 possesses an N-terminal signal peptide and a C-terminal KDEL motif responsible for ER retention. Gp96 contains several conserved domains: N-terminal nucleotide binding site, acidic domain, leucine zipper and KEKE domains, which are involved in protein–protein interaction, and a peptide binding site. The C-terminal portion is crucial for dimerisation (Li *et al*, 2002). (**C**) Vip binds to surface-exposed Gp96. Total cell extracts from surface biotin-labelled Caco-2 and L2071 cells were incubated with Vip and immunoprecipitated with anti-Vip and Sepharose beads (Pull-down Vip). In control, the incubation step with Vip was omitted (Control). To control membrane impermeability, α-catenin was immunoprecipitated using anti-α-catenin polyclonal antibody. Captured proteins were separated on a 10% polyacrylamide gel, transferred onto PVDF membrane and probed with streptavidin (Streptavidin) or with anti-Gp96 (Anti-Gp96), or with anti-α-catenin (Anti-α-cat).

pretreated with Vip or anti-Gp96 and used for invasion assays with *L. monocytogenes*. As compared to nontreated cells, preincubation of L2071 cells with both Vip or anti-Gp96 led to a reduced invasiveness in a dose-dependent manner (Figure 8A). In contrast, cell pretreatment with rat IgG or rabbit preimmune serum had no effect on bacterial invasion, whatever the dose used. Conversely, *L. monocytogenes* were preincubated with Gp96 or anti-Vip and used for invasion assays in L2071 cells. Invasiveness of bacteria preincubated both with Gp96 or anti-Vip was also significantly reduced (Figure 8B). An equivalent concentration of rat IgG or rabbit preimmune serum did not have any effect on bacterial entry. These results strongly suggest that Gp96 is acting as a Vip receptor and that the Vip–Gp96 interaction is involved in *Listeria* entry into L2071 cells.

Overexpression of Gp96 enhances Listeria entry

To confirm the role of Gp96 in *Listeria* entry, we analysed the effect on invasion of an increased expression of Gp96. L2071 cells were transfected with a pcDNA3 vector containing *gp96* cDNA (Prasadarao *et al*, 2003). Overexpression of Gp96 resulted in a significant increased entry (1.75-fold, $P<0.001$) of *L. monocytogenes* in transfected cells (Figure 9). Moreover, the increased expression of Gp96 had no effect on the invasion level of Δ*vip*, indicating that this process was Vip dependent. These results confirmed the role of the Vip–Gp96 interaction in *Listeria* entry into L2071 cells.

Vip is required for virulence

To analyse the potential role of *vip* in virulence, we performed oral inoculations of *hEcad* transgenic mice with the WT, Δ*vip* and Δ*inlA* strains (5×10^9 CFU) (Figure 10A). The number of bacteria in the small intestine, mesenteric lymph nodes, liver and spleen of mice was determined 24, 48 and 72 h after infection. In all the organs tested, bacterial counts for Δ*vip* were significantly affected as compared to the WT strain. The number of Δ*vip* bacteria was dramatically impaired in the intestine and liver 72 h after inoculation ($\approx$1.5–3 log). In lymph nodes and spleen, the virulence of Δ*vip* was also attenuated when compared to WT, but the bacterial growth

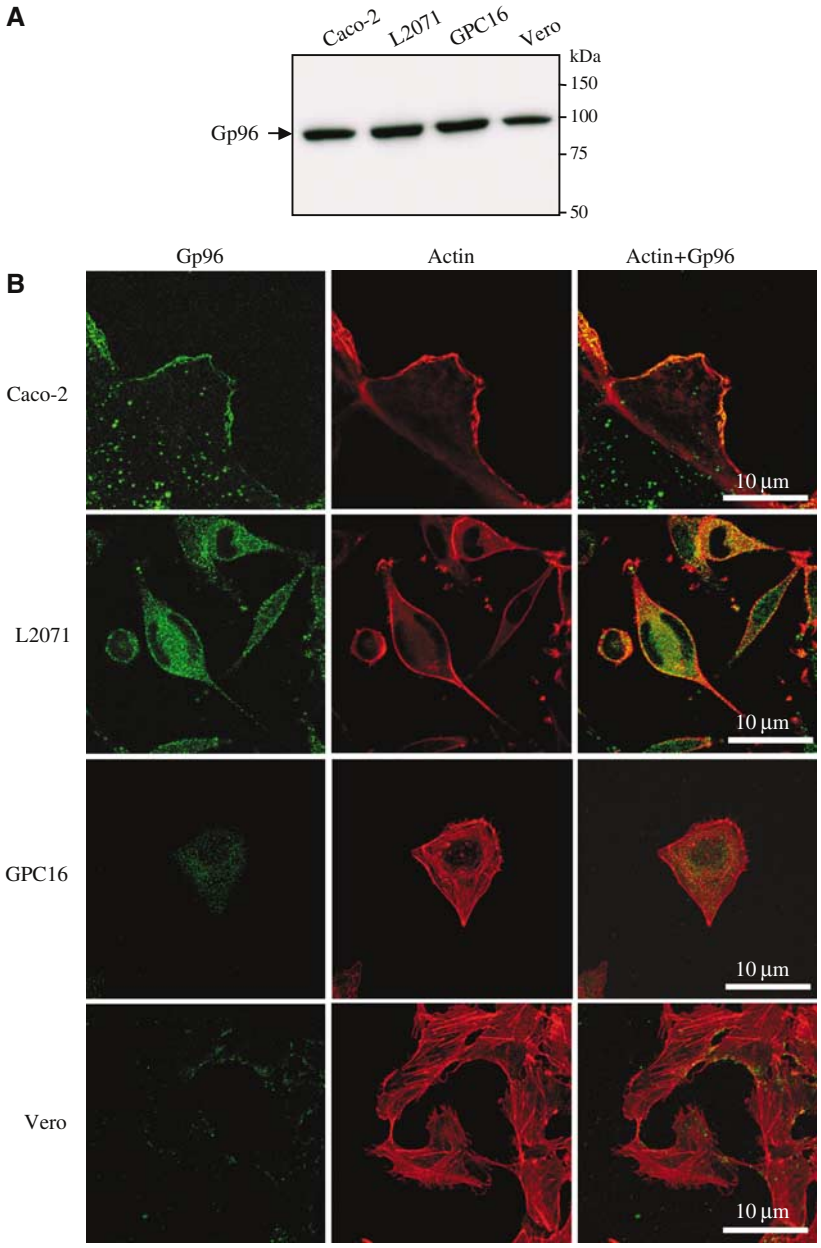

Figure 7 Gp96 is expressed at the cell membrane. (**A**) Western blot analysis of the cellular expression of Gp96. Total extracts from Caco-2, L2071, GPC16 and Vero cells were separated by SDS–PAGE and transferred onto PVDF membrane. Gp96 was revealed with an anti-Gp96. (**B**) Cellular localisation of Gp96. The distribution of Gp96 in Caco-2, L2071, GPC16 and Vero cells was analysed by confocal immunofluorescence laser microscopy. Nonpermeabilised cells were stained with anti-Gp96 and phalloidin to label F-actin.

curves were similar, indicating that the slight growth delay observed for Δ*vip* in BHI at 37°C (Figure 3B) has no consequence on bacterial multiplication in these organs. In the liver, the *vip* mutant was rapidly cleared. In all the organs tested, bacterial loads for Δ*vip* were comparable to those for Δ*inlA*. The same decrease in bacterial counts was obtained in the organs of guinea-pigs after oral inoculation of Δ*vip* as compared to WT (data not shown). These results revealed a role for Vip in *Listeria* virulence.

Nontransgenic mice were infected intravenously in order to analyse the effect of the *vip* deletion in late stages of the infection, beyond invasion of intestinal cells (Figure 10B). Mice were infected intravenously with either Δ*vip* or WT bacteria (10^4 CFU) and bacterial counts in organs determined. In contrast to what occurs with Δ*inlA* (Lecuit *et al*, 1999), infection of the spleen, liver and brain of mice with Δ*vip* was severely impaired (2–3 log) 72 h postinoculation, as in transgenic mice after oral inoculation, clearly implicating Vip in a late stage of the infectious process, that is, a role distinct from that of InlA.

To analyse if the virulence attenuation associated to *vip* deletion could be influenced by the presence in intestinal cells of an E-cadherin able to recognise InlA, we performed oral inoculation of nontransgenic mice (10^{10} CFU). As already shown (Lecuit *et al*, 2001), InlA had no significant role in infections initiated via the oral route in nontransgenic mice (Figure 10C). In contrast, the absence of Vip had a substantial effect on the bacterial counts in the organs of normal mice orally infected. Differences between Δ*vip* and WT were

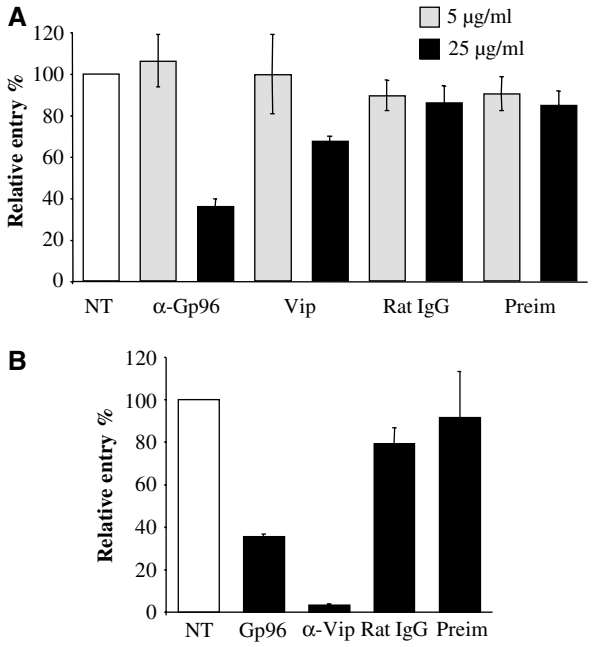

Figure 8 Saturation of bacterial Vip or cellular Gp96 blocks *L. monocytogenes* invasion. (**A**) Pretreatment of cells with anti-Gp96 or Vip blocks *Listeria* entry. L2071 cells were pretreated with 5 or 25 μg/ml of anti-Gp96 or purified Vip and used for gentamicin assays with *L. monocytogenes*. Entry of *Listeria* into treated cells was compared to those into nontreated cells (NT), or cells treated with an equivalent concentration of rat-IgG or rabbit preimmune serum (preim) as control. (**B**) Pretreatment of *Listeria* with Gp96 or anti-Vip blocks entry into cells. Bacteria were preincubated with 25 μg/ml of purified Gp96 or anti-Vip and used for gentamicin assays on L2071 cells. Entry of treated bacteria was compared to that of nontreated bacteria (NT), or bacteria treated with an equivalent concentration of rat-IgG or rabbit preimmune serum (preim) as control. Values are given relative to the invasion of the WT strain arbitrarily fixed to 100. Experiments were repeated three times in duplicate.

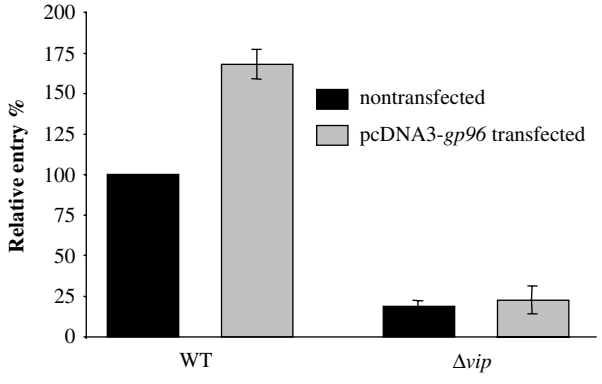

Figure 9 Overexpression of Gp96 enhances *Listeria* invasion. Entry of *L. monocytogenes* WT and Δ*vip* strains was analysed by gentamicin assay in L2071 cells and L2071 cells transfected with the pcDNA3 vector containing *gp96* cDNA. Values are given relative to the invasion of the WT strain into L2071 cells arbitrarily fixed to 100. Experiments were repeated three times in duplicate for each cell line.

strikingly comparable in *hEcad* transgenic and nontransgenic mice, indicating that the species-specific cadherin in the intestinal barrier does not influence the phenotype of the *vip* mutant and confirming the role for Vip not only at the intestine level but also in late stages of infection.

Since Vip and InlA appear to be implicated in different stages of the infection, we evaluated a potential synergy between these two virulence factors. We constructed a double *vip/inlA* mutant and performed oral inoculation of guinea-pigs (Figure 10D). At 72 h after inoculation of 10^{10} *Listeria*, bacterial loads of the *vip* or *inlA* single mutants in all organs were comparable, but severely impaired as compared to those of WT. The *vip/inlA* double mutant behaved as the two single mutants, suggesting the absence of a detectable additive effect of the two deletions.

Discussion

This study describes the identification of Vip, a novel *Listeria* virulence factor only present in pathogenic *Listeria* species, which is required for entry into some mammalian cells and virulence. We also report that the Vip receptor is Gp96, a protein already known as an ER resident chaperone and reported to control the immune response, possibly by playing a role in antigen presentation (Li *et al*, 2002).

Vip is anchored to the *Listeria* surface by SrtA, similar to InlA and at least three other LPXTG proteins (Bierne *et al*, 2002). Several LPXTG proteins are known to play a role in *Listeria* virulence. InlA promotes bacterial invasion of epithelial cells by interacting with its host receptor E-cadherin (Mengaud *et al*, 1996b) and crossing of the intestinal and placental barriers (Lecuit *et al*, 2001, 2004). Mutants deleted for the *inlGHE* locus or for *inlH* exhibit a loss of virulence in the mouse model (Raffelsbauer *et al*, 1998; Schubert *et al*, 2001). Finally, as recently shown, InlJ also plays a role in virulence (Sabet *et al*, 2005). However, except for InlA, one of the best-characterised *Listeria* virulence factors, the role of the other LPXTG proteins remains to be elucidated. Vip is not an LRR protein and thus does not belong to the internalin family. Interestingly, as all the LPXTG protein-encoding genes implicated in virulence, *vip* is absent in all nonpathogenic *Listeria* species. Moreover, *vip* is present in all *L. monocytogenes* lineage I and II that include serovars generally implicated in human disease (1/2a, 1/2b, 4b) and only absent in two rare *L. monocytogenes* serovars (4a, 4c) (Doumith *et al*, 2004).

vip is positively regulated by PrfA, the master regulator of *L. monocytogenes* virulence genes. As for three previously known PrfA-dependent virulence genes (*inlB, inlC, hpt*), *vip* was not detected and reported as regulated by PrfA in BHI at 37°C in a transcriptomic analysis (Milohanic *et al*, 2003). This is probably due to low expression in these conditions.

Vip is required for efficient entry into Caco-2 and L2071, but not into GPC16 and Vero cells. At the surface of Caco-2 and L2071 cells, Vip interacts with Gp96 and this interaction is critical for entry. Data are consistent with a direct interaction between Vip and Gp96 during entry, but protein regions involved are unknown. Vip possesses a proline-rich region. Proline-rich motifs are often involved in protein–protein interactions (Kay *et al*, 2000). The role of the proline-rich domain of Vip is unknown and could be involved in the interaction with Gp96.

Gp96 also referred to GRP94 is expressed ubiquitously. It is part of the Hsp90 protein family (Csermely *et al*, 1998), but it is an unusual member of this family, since it contains an N-terminal signal sequence and a C-terminal sequence for ER retention (Li *et al*, 2002). As shown in this report, Gp96 is not restricted to the ER in Caco-2 and L2071 cells, and is also

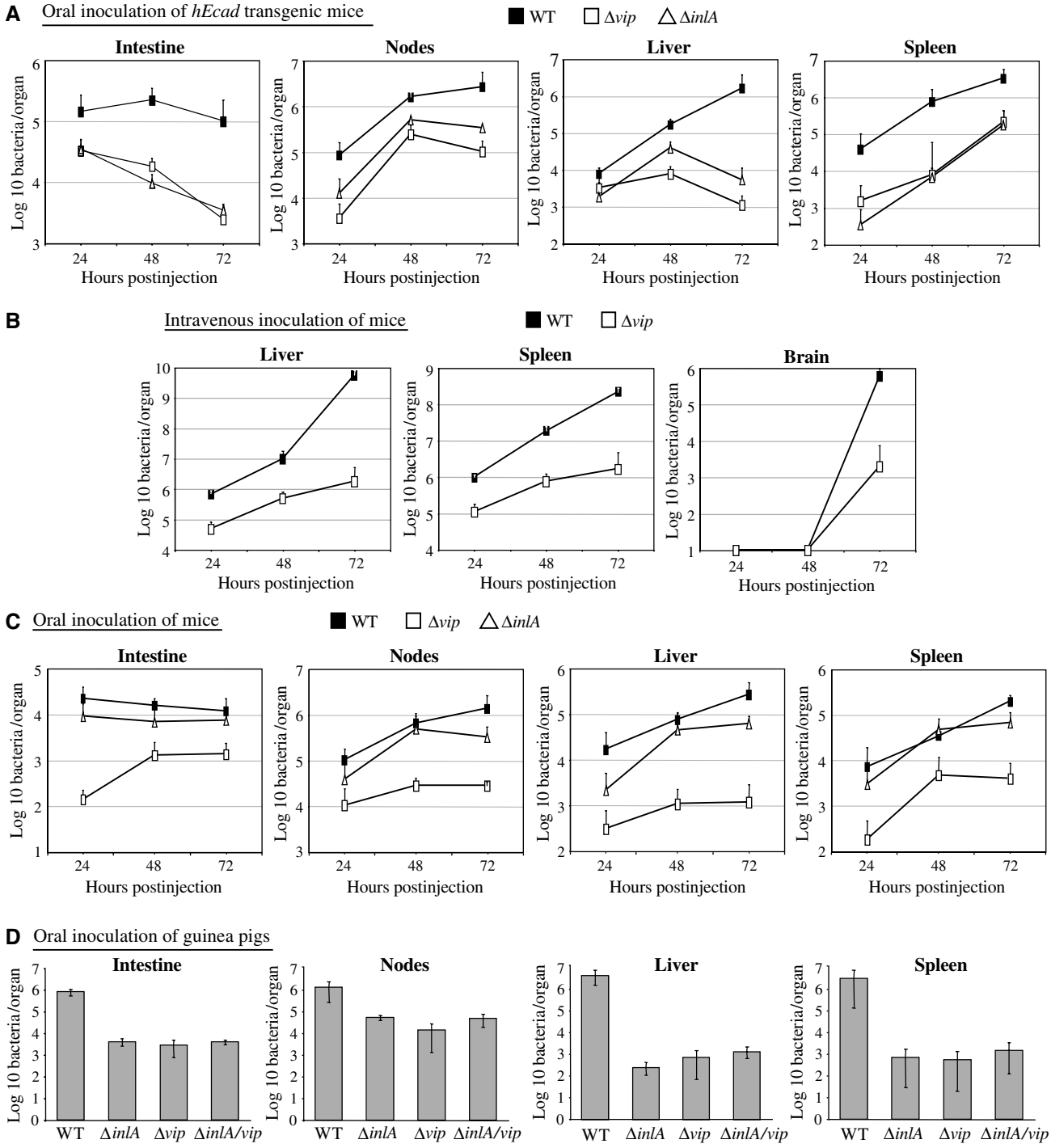

Figure 10 Vip is required for virulence. (**A**) Bacterial counts of *L. monocytogenes* WT, Δ*vip* and Δ*inlA* strains in the intestine, lymph nodes, liver and spleen of *hEcad* transgenic mice 24, 48 and 72 h after oral inoculation of 5×10^9 CFU. (**B**) Bacterial counts of *L. monocytogenes* WT and Δ*vip* strains in the liver, spleen and brain of mice 24, 48 and 72 h after intravenous inoculation of 10^4 CFU. (**C**) Bacterial counts of *L. monocytogenes* WT, Δ*vip* and Δ*inlA* strains in the intestine, lymph nodes, liver and spleen of mice 24, 48 and 72 h after oral inoculation of 5×10^9 CFU. (**D**) Bacterial counts of *L. monocytogenes* WT, Δ*vip*, Δ*inlA* or Δ*vip/inlA* strains in the intestine, lymph nodes, liver and spleen of guinea-pigs 72 h after oral inoculation of 10^{10} CFU.

detected at the cell surface. In agreement with our results Gp96 has previously been immunoprecipitated from cell surfaces and purified from plasma membrane (PM) (Srivastava *et al*, 1986; Altmeyer *et al*, 1996). In rat pancreas, it was shown to be present both in ER and PM (Takemoto *et al*, 1992). Gp96 has also been detected at the PM of the hepatocytes of porcine liver and of endothelial and Kupffer cells (de Crom *et al*, 1999). However, as we observed with GPC16 and Vero cells, the Gp96 surface expression is not a property of all cells, explaining why Vip function in entry is only detectable in cells where the receptor is expressed at the surface.

During cell invasion, *Listeria* ligands interact with and activate mammalian receptors, which results in the tight engulfment of the bacterial body by the cell membrane

(Cossart *et al*, 2003). Recently, it was shown that the outer membrane protein A (OmpA) of *Escherichia coli* K1 interacts with Ecgp, a Gp96 homologue, on human brain microvascular endothelial cell (HBMEC) (Prasadarao *et al*, 2003). OmpA-mediated *E. coli* invasion of HBMEC induces both focal adhesion kinase (FAK) and PI3Kinase activation, which likely occurs via Gp96 signalling, generating cytoskeleton rearrangement required for *E. coli* K1 entry (Reddy *et al*, 2000a; Kim, 2002). *L. monocytogenes* interaction with cells does not seem to induce FAK activation for cytoskeletal rearrangements (Reddy *et al*, 2000b; K Ireton and P Cossart, unpublished data). Vip treatment of L2071 cells results in no increase in tyrosine phosphorylation of protein associated with p85α or Gp96 (data not shown). It is thus possible that the Vip–Gp96 interaction plays a role in *Listeria* entry through other signal transduction events associated with Gp96 responses that remain to be elucidated.

Gp96 as a member of the HSP protein family, associates with peptides and proteins to facilitate folding and unfolding (Ma and Hendershot, 2001; Li *et al*, 2002). There is increasing evidence that Gp96 modulates both the innate and adaptive immune response. Gp96 has been reported to associate with intracellular peptides in particular listerial peptides (Sponaas *et al*, 2001), and to deliver associated peptides into the antigen presenting pathway, resulting in the induction of specific T cell responses. A receptor responsible for the uptake of Gp96-peptide complexes has been identified as the α_2 macroglobulin receptor CD91, expressed on professional antigen presenting cells (Li *et al*, 2002; Srivastava, 2002). Furthermore, Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway (Vabulas *et al*, 2002). Gp96 also specifically binds and activates neutrophils and monocytes (Radsak *et al*, 2003). Gp96 thus acts as a danger signal. In addition, Gp96 is crucial for Toll-like receptors subcellular localisation (Randow and Seed, 2001; Hornef *et al*, 2003). We propose that Vip would contribute to *Listeria* pathogenicity by impairing the function of Gp96, in turn reducing the host immune response.

From the intestinal lumen, *Listeria* invades the small intestine, translocates to mesenteric lymph nodes and spreads to the liver, spleen and brain. Most of the successive steps of this *in vivo* infectious process as well as the bacterial factors involved remain elusive. Several *Listeria* surface proteins have been shown to play a role in virulence (Dussurget *et al*, 2004). InlA plays a key role in the crossing of the intestinal and placental barriers (Lecuit *et al*, 2001, 2004). Recently, we showed that FbpA is necessary in a step directly downstream of the InlA site of action (Dramsi *et al*, 2004). However, infection stages in which other surface virulence factors are implicated remain unknown. Using suitable animal models and inoculation routes, we demonstrate here a role for Vip in *Listeria* virulence and establish its role in different stages of the infectious process. In contrast to FbpA, the phenotype of the *vip* mutant is not dependent on the InlA–E-cadherin interaction at the intestinal barrier, as Δ*vip* is attenuated in nontransgenic mice after oral infection. Moreover, the *vip* mutant is attenuated after intravenous infection. A similar virulence attenuation irrespective of the route of inoculation has been already observed with the *srtA* mutant (Bierne *et al*, 2002; Garandeau *et al*, 2002). As InlA plays no role in murine infections, this suggested the implication of other LPXTG proteins in listeriosis. Vip appears as one of these proteins.

In summary, *Listeria* could use Gp96 as a receptor for invasion, but also bind and sequester Gp96 to subvert the immunological response in the course of the infectious process.

Materials and methods

Bacterial strains and media

We used the following *L. monocytogenes* isogenic strains: EGDe (WT, ATCC BAA-679), EGDeΔ*prfA* (gift from Dr M Kuhn), EGDeΔ*inlA2* (Lingnau *et al*, 1995), EGDeΔ*srtA* and Δ*srtB* (Bierne *et al*, 2004). *Listeria* were grown in BHI medium (Difco) at 37°C. *E. coli* strains were grown in LB medium (Difco) at 37°C. Antibiotics were included at the following concentrations: chloramphenicol, 7 μg/ml; kanamycin, 20 μg/ml; ampicillin, 100 μg/ml.

Generation of purified Vip and anti-Vip

vip (lacking signal peptide encoding region) was amplified by PCR from chromosomal DNA using primers 2323-D2 (5′-GGAATTCCATA TGTATGAAGAGTCA-3′) and 2323-R2 (5′-CCGCTCGAGTTTTTTCCGT TTGAA-3′). PCR product was *Nde*I–*Xho*I digested and inserted in pET-22b(+) (Novagen). The construction was verified by sequencing of the insert from both junctions and used to transform *E. coli* BL21 DE3 (Novagen). The recombinant Vip-6xHis protein was obtained at 37°C in exponential cultures adding 0.1 mM IPTG for 2.5 h. The Vip-6xHis-tagged protein was purified using TALON™ -metal affinity resin (Clontech). Purified Vip-6xHis protein was concentrated using centriplus YM-10 columns (Millipore).

Purified Vip-6xHis protein (~1 mg) was sent to CRIFFA (Charles River-Laboratories, Barcelona, Spain) for immunisation of New-Zealand rabbits and production of specific anti-Vip polyclonal rabbit antibodies.

RNA techniques

RNA from *Listeria* was isolated and purified with the High Pure RNA Isolation Kit (Boehringer). RT–PCR was performed according to the protocol of the Superscript one-step RT–PCR system (Invitrogen). Oligonucleotides used were as follows: for *vip*, *lmo0320*-F (5′-TAGCCCTTTAGATACGCCC-3′) and *lmo0320*-R (5′-TAATTTGTTCCTGCACAGCG-3′); for *iap*, *iap*-F (5′-AAAGCAACT ATCGCGGCTAC-3′) and *iap*-R (5′-TCTTGAACAGAAACACCGTA-3′); for *lmo0319*, *lmo0319*-F (5′-CTTACAACGTCGATGGAAAAGG-3′) and *lmo0319*-R (5′-AGAAGTAGTTTTTGCGCTCTGC-3′); for *hly*, *hly*-F (5′-CAAACTGAAGCAAAGGATGC-3′) and *hly*-R (5′-CATACCTGGCA AATCAATGC-3′). No RT–PCR product was detected in controls lacking reverse transcriptase, demonstrating the absence of DNA contaminating the RNA preparations.

For Northern blot, quantitative detection of RNA was carried out according to the Northernmax-Gly glyoxal-based system protocol (Ambion).

Mutagenesis and complementation

A DNA fragment containing 0.5 kb of the sequence upstream of *vip* was PCR generated using *lmo0320*-MF1 (5′-CAGGAATTCCCGGAC GAACTAACCGCCG-3′) and *lmo0320*-MR1 (5′-GGGGTACCCCCATT TAAGCGGTCGTTCC-3′). The fragment was cloned into *Eco*RI–*Kpn*I-digested pOD23 (Dussurget *et al*, 2002). A DNA fragment containing 0.5 kb of the sequence downstream of *vip* was PCR generated using *lmo0320*-MF2 (5′-GCTCTAGAGCTAGTCAAAACACCGGCTC-3′) and *lmo0320*-MR2 (5′-AAAACTGCAGAGTCCTGCTTCGTTTGGC-3′). The fragment was cloned into *Xba*I–*Pst*I-digested pOD23 containing the *vip* upstream fragment, constructing pDC4. The construction was verified by sequencing. pDC4 was electroporated into *L. monocytogenes* EGDe. Allelic replacement was performed as described previously (Cabanes *et al*, 2004). The replacement was confirmed by Southern, PCR and RT–PCR. To generate a Δ*vip*-Δ*inlA* mutant, *L. monocytogenes* Δ*inlA2* was electroporated with pDC4 and replacement confirmed by PCR.

For complementation, the entire *vip* gene and flanking regions were amplified using primers *lmo0320-Bam*HI (5′-CGGGATCCA TCAAAATCCCCACGCC-3′) and *lmo0320-Xba*I (5′-GCACATGAACG TCGGTAACATTCC-3′). PCR products were *Bam*HI–*Xba*I digested and ligated to the site-specific phage integration vector pPL2 (Lauer *et al*, 2002) digested by *Bam*HI–*Spe*I, constructing pDC28. The construction was verified by sequencing. pDC28 was transformed

into *E. coli* S17-1 and the resulting strain was mated into EGDeΔ*vip*. Chloramphenicol-resistant transconjugants were tested by PCR for pDC28 integration at the appropriate chromosomal site using primers PL102 (5′-TATCAGACCAACCCAAACCTTCC-3′) and PL95 (5′-ACATAATCAGTCCAAAGTAGATGC-3′). Primers *lmo0320*-F and *lmo0320*-R were used to confirm the presence of *vip* in the complemented strain.

In vitro invasion assays

Invasivity tests were performed as described previously (Cabanes *et al*, 2004). To test the ability of Gp96 or anti-Vip to inhibit *L. monocytogenes* WT entry, bacteria were pretreated with 5 or 25 μg/ml of Gp96 (SPP-766; Stressgen), or anti-Vip, or rat IgG (Sigma), or rabbit preimmune serum for 1 h at 4°C before infection. To test the ability of Vip or anti-Gp96 to inhibit entry of *L. monocytogenes* WT, 25 μg/ml of Vip, or anti-Gp96 (SPA-850; Stressgen), or rat IgG, or rabbit preimmune serum was added to cells for 1 h at 37°C before infection. At the concentrations used, proteins or antibodies have no effect on cell viability, as determined by Trypan blue staining.

Transient transfections in L2071 cells were performed using Fugene 6 (Roche) following the manufacturer's protocol. L2071 cells were transfected with the pcDNA3 vector containing *gp96* cDNA (Prasadarao *et al*, 2003). Invasion assays were performed 24 h after transfection.

Virulence studies

Oral infections were performed as described previously (Lecuit *et al*, 2001). For mice, 5×10^9 CFU mixed with PBS 150 mg/ml $CaCO_3$ were injected intragastrically to 6- to 8-week-old female B6/SJL mice (Charles River) or *iFABP-hEcad* transgenic mice starved for 24 h. For guinea-pigs, 300 g male Hartley (Charles River) starved for 2 days were anaesthetised and injected intragastrically with PBS 25 mg/ml $CaCO_3$ followed by 10^{10} CFU. At 24, 48 or 72 h after infection, the organs were dissected. The small intestine was rinsed and incubated for 2 h in 100 mg/l gentamicin to kill extracellular bacteria from the intestinal lumen. For intravenous injection, 5- to 6-week-old female BALB/c mice (Charles River) were injected intravenously with 10^4 CFU. For bacterial numerations, the number of CFU was determined by plating dilutions of organ homogenates (four animals for each time point). Animal experiments were performed according to the Institut Pasteur guidelines for laboratory animal husbandry.

Ligand overlay assay

Cells were lysed in NP-40 buffer (20 mM Tris pH 8.0, 1% (v/v) NP-40, 137 mM NaCl, 10% (v/v) glycerol and protease inhibitors cocktail). Lysates were clarified and protein concentrations determined. A 40 μg portion of solubilised proteins was separated on a 10% polyacrylamide gel, electroblotted onto PVDF membrane and blocked overnight in 20 mM Tris pH 7.5, 0.9% NaCl and 0.1% Triton X-100 containing 3% milk. The membrane was incubated with 50 μg/ml of Vip, washed, incubated with anti-Vip, then with anti-rabbit HRP conjugate. Bound proteins were detected using the ECL detection kit (Amersham). Proteins were identified by mass spectrometry as described previously (Cabanes *et al*, 2004).

Pull-down assay

For cell surface protein biotinylation, live cells were washed and incubated with 1 mM sulpho-NHS biotin for 30 min at 4°C. Sulpho-NHS biotin was quenched with PBS 100 mM glycine. Cell lysates were prepared as described above. A 400 μg portion of total protein extracts was incubated with 60 μl of a 20% (v/v) suspension of Protein G Sepharose beads (Amersham) for 2 h at 4°C. The beads were sedimented and the supernatant incubated with 10 μg of purified Vip at 4°C overnight. Vip was immunoprecipitated by addition of 5 μl of anti-Vip 2 h at 4°C and then with 60 μl of Protein G Sepharose beads for 2 h at 4°C. Beads were sedimented and washed five times. To control membrane impermeability, α-catenin was immunoprecipitated using anti-α-catenin polyclonal antibody (H-297; Santa Cruz Biotech). Immunoprecipitated proteins were eluted and boiled in Laemmli buffer containing 0.1 mM DTT and 5% β-mercaptoethanol. Samples were analysed by SDS–PAGE, immunoblotted with primary antibody and with HRP conjugate, or with HRP-streptavidin. Bound proteins were detected using the ECL detection kit.

Immunofluorescence analysis

Bacteria were fixed with 3% paraformaldehyde, washed and stained with the anti-Vip antibody diluted 1:200 and Alexa Fluor 488-conjugated goat anti-rabbit (Molecular Probes) diluted 1:200. InlA was stained by mAbs anti-InlA L7.7 (Mengaud *et al*, 1996a) diluted 1:400 and Alexa Fluor 546-conjugated goat anti-mouse (Molecular Probes) diluted 1:200. Cells were fixed with 3.5% paraformaldehyde and blocked in PBS containing 1% BSA and 1% gelatin. Cells were stained with anti-Gp96 (H-212; Santa Cruz Biotech) diluted 1:100 and Alexa Fluor 488-conjugated goat anti-rabbit (Molecular Probes) diluted 1:200. Alexa-Phalloidin-546 (Molecular Probes) diluted 1:200 was used to label actin filaments. Preparations were observed with a confocal laser scanning microscope (Zeiss LSM 510). Analyses shown are representative of data obtained from an average of 2–4 independent analysis.

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